

REMARKS

A. Regarding the Amendments

Pursuant to the Restriction Requirement, claims 12 and 25-31 are cancelled herein without disclaimer and without prejudice to Applicants' pursuing prosecution of subject matter encompassed within the claims in an application claiming benefit of priority of the subject application. Claim 24 has been amended as set forth in the attached "Version With Markings To Show Changes Made." Specifically, support for claim 24 may be found at page 14, lines 9-17, and the definition of "TGF- β " is known in the art. As amended, the claims are supported by the specification and the original claims. Applicants submit that the amendments to the claims are for clarity and should not be construed as amendments affecting patentability under Festo Corp. v. Shoketsu Kinzoku Kogyo Kabushiki Co., 234 F.3d 558, 56 USPQ2d 1865 (Fed. Cir. 2000) (en banc). Thus, upon entry of the amendments, claims 10, 18-24 will be pending.

B. Rejection Under 35 U.S.C. § 112

Applicants respectfully traverse the rejection of claims 10 and 18-24 under 35 U.S.C. § 112, first paragraph, as containing subject matter allegedly not described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention. It is alleged in Paper No. 8 that claims 10 and 18-24 are not enabled for a DNA composition useful in inducing immune protection against arthritogenic peptides in a host comprising a recombinant gene expression vector which encodes bacterial dnaJp1 peptide. Applicants respectfully disagree. The test of enablement is whether one of skill in the art could make or use the claimed invention without undue experimentation. In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). It is respectfully submitted that undue experimentation would not have been required to practice the claimed invention.

In particular, it is stated in Paper No. 8 that the specification does not teach a skilled artisan how to administer the claimed compositions for immune protection. Applicants

respectfully disagree. The Examiner's attention is respectfully drawn to pages 29-30, under the heading "THERAPEUTIC METHODS OF THE INVENTION" and the subheading "Methods for Use of the Vaccines of the Invention." On page 29, line 21 through page 30, line 8, specific methods of administration of the nucleotide vaccines of the invention are discussed. This section discloses that the preferred method of administration is intradermal, but that methods such as injection, absorption or transdermal transmission across the skin or mucosa of the host may also be used.

It is also asserted in Paper No. 8 that "the specification fails to provide an enabling disclosure for the preparation and use of a DNA composition," and that gene therapy is a limited technology. Applicants respectfully submit that the specification teaches preparation of the claimed composition and administration, preferably intradermal (i.d.) administration, for example, at page 29, line 21 through page 30, line 8, such that the skilled artisan would have known how to make and use the claimed compositions.

In support of Applicants' position that the specification would have enabled use of a claimed composition, La Cava, et al. *J. Immunol.* 164(3):1340-5, Feb. 1, 2000 is submitted herewith (Exhibit A). La Cava, et al. describe that i.d. administration of DNA in one location is transported to a second location, and an immune response is obtained in the second location. In the "Materials and Methods" section of La Cava, et al., the DNA is inserted in a p290 expression vector. It is noted that, while La Cava, et al. was published in February 2000, it is recognized that later publications can be used as evidence of the state of the art existing at the time of filing of an application. In re Hogan, 559 F.2d 595, 605, 194 USPQ 527, 537 (CCPA 1977). The specification discloses the state of the art as being able to generate an immune response using the claimed composition by, for example, i.d. administration. As such, it is submitted that La Cava, et al. confirm that one skilled in the art would have been able to make and use a composition of the invention, for example, for i.d. administration of recombinant gene expression vectors for inducing an immune response.

The disclosure in the specification is referred to in Paper No. 8 as a “paper protocol.” Applicants respectfully submit that this disclosure is sufficient to enable one of skill in the art to administer the compositions of the claimed invention. Experimental illustration of every embodiment is not required, if the invention is otherwise disclosed in a manner such that one skilled in the art will be able to practice it without an undue amount of experimentation. In re Borkowski, 422 F.2d 904, 908, 164 USPQ 642, 645 (CCPA 1970).

In support of the position that the specification does not teach how to practice the claimed invention, it is asserted that Applicants have not “shown or disclosed a correlation between *in vitro* and *in vivo* studies or that there are animal models that correlate to human (i.e. person) efficacy.” (Paper No. 8, page 3.) Applicants respectfully submit that this assertion is contrary to the examination guidelines set forth in the MPEP 2107.02, which states, “[e]vidence does not have to be in the form of data from an art-recognized animal model for the particular disease or disease condition to which the asserted utility relates. Data from any test that the applicant reasonably correlates to the asserted utility should be evaluated substantively... Office personnel should be careful not to find evidence unpersuasive simply because no animal model for the human disease condition had been established prior to the filing of the application.”

It is respectfully submitted that it is not necessary to show correlation between *in vitro* and *in vivo* studies or to show animal models that correlate to human efficacy in the claimed invention. The specification provides sufficient guidance to practice the invention. It is submitted that working examples are not required where the invention is otherwise disclosed in a manner such that one skilled in the art would have been able to practice it without an undue amount of experimentation. As confirmed by La Cava, et al., one of skill in the art would have been able to practice the present invention as disclosed.

It is also asserted in Paper No. 8 that the “specification fails to provide guidance to the skilled artisan on the parameters for DNA vaccine for the breadth of the claimed invention.” (Paper No. 8, page 4.) Applicants respectfully submit that the specification, with the knowledge

of one skilled in the art at the time of filing the application, provides the necessary guidance to perform the claimed invention. Specifically, it is alleged in Paper No. 8 that one of skill in the art would need to engage in undue experimentation regarding: the fate of the vector, the trafficking of the genetic material within cellular organelles, the rate of degradation of DNA, the level of mRNA produced, the stability of the mRNA produced, the amount and stability of the protein produced, and the protein's compartmentalization within the cell, or its secretory fate, once produced.

Applicants respectfully submit that the present specification discloses specific guidelines for development of the claimed compositions, for example, composition of the vaccine, including the polynucleotides and specific vectors, the structure of dnaJp1 (SEQ ID No: 4) and methods for obtaining polynucleotides encoding dnaJp1. The specification also discloses how to identify individuals for whom the claimed compositions will be efficacious, methods of administration and therapeutic methods of using the compositions. One method of administration, as supported by La Cava, et al., is i.d. administration of the claimed compositions. Little further experimentation, none of it undue, is left to the artisan. It is routine in the art to perform clinical trials to determine details such as, but not limited to, dosage and routes of administration.

The Examiner has also alleged in Paper No. 8 that there are no working examples of the claimed invention. It is submitted that working examples are not required where the invention is otherwise disclosed in a manner such that one skilled in the art would have been able to practice it without an undue amount of experimentation. As set forth above, the specification provides sufficient guidance to practice the invention, including details regarding the polynucleotides and specific vectors, the structure of dnaJp1 (SEQ ID No: 4), methods for obtaining polynucleotides encoding dnaJp1, how to identify individuals for whom the claimed vaccine will be efficacious, and therapeutic methods of using the compositions are all set forth in the specification.

The Examiner also states that the specification does not provide any guidance as to how to make and use genetic constructs which would produce the desired effect. Applicants

respectfully disagree. Pages 14-18 discuss, in detail, the nucleotide vaccines of the invention, specifically selection of the polynucleotides, selection of the vector and potential other necessary materials such as "helper cell lines" and carriers. It is further asserted by the Examiner that even if an effective genetic material is constructed, that it is not evident that enough cells can be transfected to provide any therapeutic benefit. However, it is submitted that the specification discloses administration of DNA-containing compounds to be administered by, for example, i.d. administration for stimulation of immune responses. As set forth above, La Cava, et al. confirm the teaching of the specification. Accordingly, it was known at the time of filing of the present application that a therapeutic effect can be derived from administration of a DNA vaccine.

Further, it is asserted that "several recent reviews indicate that efficient delivery and expression of foreign DNA has not yet been achieved by any method." (Paper No. 8, page 4.) Initially, it is noted that the present invention is directed to vaccines, used to attain an immune response, rather than gene therapy for prolonged expression used, for example, as disease treatment. The Examiner's attention is respectfully drawn to the specification of the present invention at, for example, page 29, line 21 to page 30, line 5, which discloses methods of administration of the compounds of the invention. La Cava, et al., confirm this disclosure by showing active uptake by both bone-marrow derived and phagocytic cells and transportation of intradermally injected DNA to remote sites. Accordingly, it is respectfully submitted that efficient delivery and expression of foreign DNA has been achieved and had been achieved as of the time of filing of the claimed invention.

Finally, it is asserted in Paper No. 8 that it is unclear in the art what the etiological cause of RA is and therefore it would be unclear and an undue burden to a skilled artisan to determine which type of composition to administer to a person with RA to reduce its exposure or predisposition to develop RA. (Paper No. 8, page 5-6.) As support for this allegation, the Examiner cites U.S. Patent No. 5,310,732, filed February 19, 1992 and PCT application WO 90/14835, published December 13, 1990. These references give the state of the art 4 and 6 years,

respectively, before the claimed invention's priority date of 1996. It is respectfully submitted that the inventors of the present invention have performed the needed experimentation and have developed new compositions to administer to a person with RA to reduce its exposure or predisposition to develop RA. The disclosed preferred method of administration is i.d., which is confirmed by La Cava, et al. to be a successful method of administration of gene expression vectors. The fact that an invention has not been previously performed in the art is not reason to find lack of enablement when the invention is made.

As such, one of skill in the art would have been able to practice the present invention, as the amended claims are directed to compositions for stimulating an immune response. The specification discloses how to use the claimed compositions for stimulating an immune response, such that one of skill in the art would have known how to use the claimed compositions, for example, by intradermal administration. La Cava, et al. confirm that one reading the specification would have been able to practice the claimed invention as disclosed. As such, claims 10, 18, 19 and 22-24 meet the enablement requirement of 35 U.S.C. §112, first paragraph. Accordingly, removal of the rejection is requested.

Applicants respectfully traverse the rejection of claims 10 and 18-24 under 35 U.S.C. §112, second paragraph as allegedly indefinite for failing to point out and distinctly claim the subject matter of the invention. Applicants respectfully submit that the explanation set forth below and the amendments to the specification overcome these rejections as illustrated below.

Claims 10, 18, 19, 20, 21 and 22 are rejected for containing the abbreviations "dnaJ" and "dnaJp1." With regard to the term "dnaJ," it is respectfully submitted that this term is well known in the art to refer to an arithritogenic peptide that is a bacterial heat-shock gene. It is noted that an internet search reveals the common usage of the term "dnaJ." A search on Google.com using the key words "dnaJ" and "gene" resulted in approximately 3,710 hits. These results reveal the common knowledge of "dnaJ" as a gene which possesses the function of ensuring viability at high temperatures. As "dnaJ" is not an abbreviation, but is the name given

to the gene, it is respectfully submitted that no further clarification is necessary. With regard to the term "dnaJp1," the Examiner's attention is respectfully drawn to page 5 of the specification, at lines 8-9, where it is stated, "one dnaJ peptide in particular (hereinafter, "dnaJp1"; SEQ ID No. 4) has been found to..." Accordingly, the term "dnaJp1" is clearly set forth in the specification. The term is also clearly specified in the claims as "bacterial dnaJp1 peptide having the amino acid sequence of SEQ ID NO: 4." As with "dnaJ," the term is not an abbreviation of any other word, but is clear as defined. As such, the terms "dnaJ" and "dnaJp1" as set forth in the specification and as used in the claims are definite. Accordingly, the terms "dnaJ" and "dnaJp1" will be clear to one skilled in the art reading the claims, and, therefore, claims 10, 18, 19, 20, 21 and 22 meet the definiteness requirement of 35 U.S.C. §112, second paragraph. Accordingly, removal of the rejection is requested.

Claim 24 is rejected for containing the term "TGF- β ." As amended, claim 24 contains both the full text and abbreviation of the compound, transforming growth factor- β (TGF- β). Accordingly, removal of the rejection is respectfully requested.

Claims 20 and 21 are rejected as redundant of claims 18 and 19, respectively. Applicants respectfully traverse the rejection. The Examiner's attention is respectfully drawn to the language of the claims. Claim 18 recites the recombinant gene expression vector of claim 10, where the vector contains at least one dnaJ polypeptide in addition to dnaJp1 polypeptide. Conversely, claim 20 recites the composition of claim 10 further comprising an additional vector where the vector contains at least one dnaJ polypeptide in addition to dnaJp1 polypeptide. Therefore, the composition resulting from claim 18 may contain only a single vector, whereas the composition of claim 20 contains no less than two vectors. Claims 19 and 21 are dependent upon claims 18 and 20, respectively, and therefore are not redundant where the claims they depend from are not redundant of one another. As claims 18 and 20 are not redundant, removal of the rejection is respectfully requested.

In re Application of:
Carson and Albani
Application No.: 09/616,247
Filed: July 14, 2000
Page 10

PATENT
Attorney Docket No.: UCSD1370-5

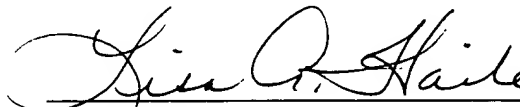
CONCLUSION

In summary, for the reasons set forth herein, Applicants maintain that claims 10, 18-24 clearly and patentably define the invention, respectfully request that the Examiner reconsider the various grounds set forth in the Office Action, and respectfully request the allowance of the claims which are now pending.

If the Examiner would like to discuss any of the issues raised in the Office Action, Applicant's representative can be reached at (858) 677-1456. Please charge any additional fees, or make any credits, to Deposit Account No. 50-1355.

Respectfully submitted,

Date: April 16, 2002



Lisa A. Haile, J.D., Ph.D.
Registration No. 38,347
Telephone: (858) 677-1456
Facsimile: (858) 677-1465

GRAY CARY WARE & FREIDENRICH LLP
4365 Executive Drive, Suite 1100
San Diego, California 92121-2133
USPTO Customer Number 28213

In re Application of:

Carson and Albani

Application No.: 09/616,247

Filed: July 14, 2000

Version with Markings - Page 1

PATENT

Attorney Docket No.: UCSD1370-5

VERSION WITH MARKINGS TO SHOW CHANGES MADE

24. (Twice Amended) The composition of claim 23, wherein the immunomodulatory compound is transforming growth factor- β (TGF- β) [TGF- β].

Cell-Mediated DNA Transport Between Distant Inflammatory Sites Following Intradermal DNA Immunization in the Presence of Adjuvant¹

Antonio La Cava,^{2,*} Rosario Billetta,^{*†} Guido Gaietta,[‡] Dustan B. Bonnin,^{*} Stephen M. Baird,[§] and Salvatore Albani^{¶||}

After intradermal genetic immunization, naked DNA is transported from the site of injection to regional lymph nodes. Little is known on how inflammation influences this process and whether DNA is transported beyond local lymph nodes. In the experiments herein reported, we injected naked DNA in the presence of adjuvant to address questions related to 1) the fate of naked DNA in the presence of inflammation; 2) the generation of immune responses to the encoded protein during inflammation; and, more in general, 3) the fate of ingested molecules beyond regional lymph nodes during inflammation. Two sites of inflammation were induced in vivo in mice. Naked DNA was injected in the nape together with adjuvant, and adjuvant only was injected at a distant peritoneal site. Injected DNA, uptaken at the primary dermal site of inflammation, was transported beyond regional lymph nodes to distant organs such as the spleen and to the distant peritoneal site of inflammation. This transport, mediated by CD11b⁺ cells, was cumulative during chronic inflammation. These results indicate a novel route of transport of DNA beyond regional lymph nodes and may have specific implications for DNA-based immune modulation. *The Journal of Immunology*, 2000, 164: 1340–1345.

In vivo injection of naked DNA elicits strong immune responses against the encoded Ag (1). Hence, genetic immunization has been used to generate protective humoral and cell-mediated immune responses (2, 3) in a wide variety of pre-clinical animal models for infectious diseases, allergy, cancer, and autoimmunity (4).

It is currently accepted that genetic immunization is based on the in situ transfection of cells whose identity varies in relation to both the method (5, 6) and the site of injection, i.e., the muscle vs the skin (7, 8). Muscle cells are transfected following i.m. injection of DNA (9, 10). Keratinocytes, fibroblasts, and dendritic cells are transfected after intradermal (i.d.)³ immunization (11–14). Bone marrow-derived APC and keratinocytes are directly transfected after gene gun immunization (15, 16). It has been suggested that the strong and persistent humoral and cellular immune responses that follow i.d. DNA immunization can be related to the important

immune surveillance functions of the skin and the skin-associated lymphoid tissues (17).

Cells of the dendritic lineage such as the bone marrow-derived Langerhans cells (LC) constitute about 5% of epidermal cells. Along with skin macrophages, LC are specialized in uptaking foreign Ags for transport to draining lymph nodes, where primary immune responses are initiated (18, 19). LC efficiently phagocytose both naked DNA and proteic Ags and are a major APC population in the immune response that follows i.d. genetic immunization (15, 20). Once transfected, skin-derived dendritic cells convey the uptaken Ag to draining lymph nodes (13) for elaboration of primary immune responses (21). Similarly, transfected macrophages can transport in vivo-phagocytosed Ag to sites distant from injection (22). However, it remains unclear whether DNA uptaken at a site of i.d. injection is transported beyond regional lymph nodes during inflammation. Such information could have significant implications for a better knowledge of the immune responses after DNA vaccination.

In the experiments herein described, we induced two distant sites of inflammation in vivo in mice to study the effect of proinflammatory chemotactic stimuli on the transport of injected DNA-based Ag. The primary site of inflammation was produced by i.d. coinjection in the nape of adjuvant with naked DNA. The second inflammatory site was induced by injection of adjuvant only at a distant peritoneal site. By combining construct-specific PCR with in situ hybridization and FACS, it was possible to follow, at the individual cell level, active cell-mediated transport of plasmid DNA between the two inflammatory sites. In situ hybridization and immunofluorescence studies detected the presence of plasmid DNA at the dermal site of inflammation for up to 6 wk after immunization and in situ expression of the encoded proteic product in cells of the epidermis and dermis. Injected DNA, uptaken at the dermal site of inflammation, could be transported beyond regional lymph nodes and detected in organs distant from the site of injection such as in the spleen. Repetitive administration at the primary inflammatory site led to accumulation of injected DNA at the distant site of inflammation. This transport was mediated by CD11b⁺

Departments of *Medicine, ²Neuroscience, ³Pathology, and ⁴Pediatrics, University of California at San Diego, La Jolla, CA 92093; ⁵Instituto de Ciencias Biomedicas, Immunology Program, School of Medicine, University of Chile, Santiago, Chile; and ⁶Androclous Therapeutics, Catania, Italy

Received for publication January 19, 1999. Accepted for publication November 17, 1999.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This study was supported in part by a grant from the Arthritis National Research Foundation and by Grants AR40770, AI37232, AR44850, AR92241, and AR41897 from the National Institutes of Health. A.L.C. is supported by a Juvenile Diabetes Foundation International Fellowship.

² Address correspondence and reprint requests to Dr. Antonio La Cava at his current address: The Scripps Research Institute, Department of Immunology-IMM23, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail: lacava@scripps.edu

³ Abbreviations used in this paper: i.d., intradermal; LC, Langerhans cells; FISH, fluorescence in situ hybridization; amp^r, ampicillin resistance gene; PEC, peritoneal exudate cell.

cells and was cumulative during chronic inflammation. The implications of this pathway for genetic immunotherapy and vaccination are discussed.

Materials and Methods

Plasmid construct

A segment of 426 b.p. (position 2995–3385) of EBV Balf2 gene was amplified from genomic DNA of EBV (strain B-958) with the primers: 5'-GCCCAAGCTTGGGATGACCATATGAGATT-3' and 5'-GCGG GATCCCGCTAGACCAGAGTCC-3' in an Ericomp EasyCycler (San Diego, CA). PCR conditions were: 1 min denaturation at 95°C; 1 min 30 s annealing at 49°C for the first 5 cycles, and 1 min at 52°C during the remaining 30 cycles; 2 min amplification at 72°C and 6 min for the last extension cycle. The amplified DNA fragment was subcloned between the *HindIII*/*BamHI* sites of the eukaryotic expression vector p290 (a gift of Dr. Tyler Parr, University of Southern California, Los Angeles, CA), which contains a CMV promoter upstream of the polylinker. p290 does not replicate in mammalian cells nor does integrate in their genome. Bacterial methylation of p290:Balf2 construct was assayed by digestion with *HpaII* and *MspI* restriction enzyme isoschizomers (23).

Genetic immunization

A total of 100 µg of p290:Balf2 DNA construct dissolved in sterile endotoxin-free water were mixed or not with 100 µg of the DNase-free synthetic adjuvant Adjuprime (Pierce, Rockford, IL) and injected i.d. into the nape of BALB/c mice (Jackson ImmunoResearch, West Grove, PA). Then, 100 µg of Adjuprime only were injected into the same mice i.p. Injections were performed, when required by the protocol, at weekly intervals. Sets of experiments were performed and confirmed at least three times.

Hematoxylin/eosin staining

Hematoxylin/eosin staining of cryosections of skin at the site of injection was performed using standard procedures, as described (24).

Fluorescence in situ hybridization (FISH)

Slides were treated with 30 µl of 100 µg/ml proteinase K (Sigma, St. Louis, MO) for 30 min at 37°C and then fixed in formaldehyde for 5 min at 4°C. A Balf2 gene probe corresponding to positions 3144–3202 was 3' tailed with TdT (Boehringer Mannheim, Indianapolis, IN) and 0.05 mM digoxigenin-dUTP (Boehringer Mannheim) for 15 min at 37°C, in 5 mM CoCl₂ and 0.5 mM dATP. An irrelevant DNA probe of the same length was tailed in the same way and used as a negative control. Reaction mixtures were spread onto glass slides and incubated at 42°C overnight. After washes with SSC and blocking with PBS/2% casein, FITC anti-digoxigenin (Boehringer Mannheim) was added 1:500, at room temperature, for 30 min.

PCR

Total DNA, isolated and purified from PBMC, spleen, and skin by Easy DNA kit (Invitrogen, Carlsbad, CA), was quantified by DNA Dipstick (Invitrogen). Then, 40 ng of total DNAs were used as templates for PCR with the conditions described above. A second round of PCR amplification was performed with identical conditions on one-tenth of the amount of the first PCR product.

For semiquantitative PCR, two independent PCR were performed, each on DNA from 5×10^5 PBMC harvested at the second week of immunization on a Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) gradient. One PCR was conducted with primers for Balf2, and the other PCR with primers for the ampicillin resistance gene (*amp*^r): 5'-GGCTCCAGATTAT CAGCAATAAACCA-3' and 5'-ATACATATTCTCAGAATGACTT GGT-3'. A single copy of the *amp*^r gene is present in the plasmid construct containing the Balf2 gene. The length of Balf2 and of *amp*^r PCR products are comparable. Starting from 25 ng, 1:2 scalar dilutions of p290 plasmid DNA were used as control templates for the PCR on *amp*^r. Intensity of *amp*^r bands was compared with the intensity of the Balf2:PCR product on a 2% ethidium-bromide-stained agarose gel.

Southern blotting

Total DNA was extracted with the Easy DNA kit (Invitrogen) according to the manufacturer's instructions. Balf2-specific PCR products were blotted on Hybond N membrane (Amersham, Buckinghamshire, U.K.). A digoxigenin-labeled synthetic oligonucleotide corresponding to the positions 3144–3202 of the Balf2 gene was used as a probe. The membrane was hybridized at 42°C overnight, then washed in 0.1% SSC/0.1% SDS,

blocked for 30 min with TBS/2% casein (Sigma), and incubated at room temperature for 30 min with AP-conjugated anti-digoxigenin Ab (Boehringer Mannheim) diluted 1:1000. After washes and incubation with the nonradioactive substrate Lumi-Phos (Boehringer Mannheim), the membrane was exposed to x-ray film (Kodak, Rochester, NY).

Confocal microscopy

Cryosections of skin at the site of injection were fixed in 2% paraformaldehyde/PBS for 10 min at room temperature, permeabilized with 0.2% Triton X-100/PBS for 10 min, and then blocked with PBS/0.005% BSA (Sigma). Tissue sections were double stained with phalloidin-FITC (Sigma) and anti-Balf2 rabbit antiserum plus rhodamine anti-rabbit Ab (H+L) Ab (Cappel ICN, Costa Mesa, CA) in 0.005% BSA. Sections were examined with a Bio-Rad MRC 1024 Laser scanning confocal system (Bio-Rad, Hercules, CA) coupled to a Zeiss Axiovert 35 M microscope (Zeiss AG, Oberkochen, Germany). Individual images (1024 × 1024 pixels) were saved to optical disk (Pinnacle Micro, Irvine, CA), converted to PICT format, and merged as pseudocolor images with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Individual-cell PCR/FISH/flow cytometry

PBMC were purified on Ficoll-Hypaque gradient (Pharmacia). Monocytes were further separated by FACS by anti-CD11b Ab (PharMingen, San Diego, CA), fixed in PBS/2% paraformaldehyde, and permeabilized with streptolysin O (Sigma) at 37°C for 30 min. Cells were pelleted and resuspended in PCR mix. PCR was performed as described above. As a positive control, primers for G3PDH were used. A digoxigenin-labeled irrelevant probe was used as a negative control. After cell pelleting and washes in 2× SSC and PBS, FISH was performed on cells in suspension. After incubation in PBS/2% BSA for 30 min at 4°C, FITC anti-digoxigenin Ab (Boehringer Mannheim) was added 1:500 v/v for 30 min at 4°C. After washes, flow cytometry was performed on a FACScan cell sorter (Becton Dickinson, San Jose, CA).

Results

Uptake of i.d. injected DNA and in vivo expression of its encoded product

Six BALB/c mice were immunized i.d. in the nape with 100 µg of naked DNA construct (p290:Balf2) plus 100 µg of the adjuvant Adjuprime. The presence of the Balf2 gene and the expression of the encoded protein product were then monitored at the site of injection. p290:Balf2 construct was uptaken in vivo and could be visualized by FISH at the dermal site of inflammation (Fig. 1) for up to 6 wk after DNA immunization (not shown). Histological examination of the same area showed cellular patchy infiltration of the dermis consistent with nonspecific inflammatory reaction (not shown).

In addition to the presence of plasmid DNA in the dermis at the site of injection of immunized animals, in situ expression of the encoded Balf2 proteic product was detected in the epidermis (not shown) and in the underlying dermis (Fig. 2), confirming recent similar observations by others (22, 25). In particular, confocal microscopy studies indicated intracellular expression of Balf2 protein in cells with the morphology of keratinocytes of the granular layer of epidermis and phagocytic cells of the dermis. Control sections incubated with rabbit preimmune serum stained negative for the presence of Balf2 (not shown).

DNA transport from the site of injection to distant sites

To follow the fate of the DNA molecules uptaken at the i.d. site of inflammation, total DNA was extracted from four injected animals from the skin of the neck at the site of injection, from PBMC, from spleen, and from the skin of the tail, which is a site distant from DNA injection. Balf2-specific PCR in individual mice showed the presence of injected DNA construct in all these tissues, except the skin of the tail (Fig. 3). The amount of transported plasmid construct was about 40 pg/10⁵ PBMC, as determined by semiquantitative PCR analysis (not shown). This transport was cell mediated, as indicated by intracellular colocalization of the construct and of



FIGURE 1. PCR-FISH for Balf2 at the i.d. site of injection. Sections taken 3 days after the last of three immunizations performed at weekly intervals. Magnification, $\times 400$.

its encoded product and by the fact that PCR on serum of immunized animals tested negative (not shown).

To rule out any potential effect of the plasmid and/or genetic construct on the DNA transport, a second set of four mice was injected with a PCR-amplified gene construct encoding for the streptococcal protein M5, and a third set of four mice with a PCR-amplified gene construct encoding the region 1495–1935 of EBV Balf2. A different eukaryotic expression vector was also employed (pRC/CMV; Invitrogen). In all such cases, PCR amplification of relevant DNA fragments revealed the presence of injected DNA in PBMC and spleen (not shown).

Cell-mediated transport of DNA between distant inflammatory sites

To address the possibility that ingested DNA molecules could be transported between two distant inflammatory sites, two simultaneous foci of inflammation were generated in the same animal of a group of six. The primary site of inflammation was induced by immunizing mice with naked DNA plus adjuvant i.d. in the neck, and a second site was induced by concomitant injection of adjuvant only at a distant peritoneal site. Peritoneal exudate cells (PEC) were collected by lavage 3 days after the third of three i.d. immunizations associated with i.p. injection of adjuvant performed at weekly intervals. Total DNA was extracted from PEC and amplified by Balf2-specific PCR. Southern blotting on PCR products with an internal Balf2 probe indicated cell-mediated transport of

FIGURE 2. Intracellular colocalization of actin filaments and Balf2 protein in the dermis at the i.d. site of DNA injection. Immunofluorescence staining indicates intracellular expression of Balf2 protein in phagocytic cells of the dermis surrounding hair follicles and blood vessels. *A*, Skin sections of the dermis labeled with anti-Balf2 rabbit antiserum and rhodamine anti-rabbit Ab. *B*, Staining of intracellular actin filaments in the same sections with phalloidin-FITC. *C*, Combined image of *A* and *B*. Arrows indicate colocalization areas in yellow-orange. Magnification, $\times 630$.

the DNA-based Ag from the dermal site of injection to the peritoneal site of inflammation (Fig. 4). Intradermal injection in the nape of mice with adjuvant only tested negative, whereas lack of inflammation at the distant site was associated with diminished DNA transport (Fig. 5).

Cumulative effect of chronic inflammation on the transport of DNA

To evaluate the effect of repetitive administration of naked DNA during inflammation, three groups of three mice were immunized i.d. in the neck with naked DNA construct at weekly intervals. Inflammation was induced in both neck and peritoneum by concomitant injection of adjuvant in both sites. One mouse from each

FIGURE 3. PCR for Balf2 on total DNA extracted from PBMC before the first DNA immunization (*lane 1*) and from the same mouse 3 days after the last of three i.d. DNA immunizations (*lane 3*) performed at weekly intervals. *Lane 2*, skin of the tail; *lane 4*, spleen; *lane 5*, dermis of the nape. The arrow points at the PCR-amplified Balf2 product. One representative experiment of three.

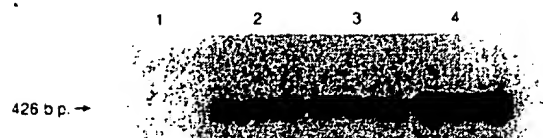


FIGURE 4. Southern blot on Balf2-PCR on total DNA from peritoneal lavage cells at the site of secondary inflammation. Lanes 2, 3, and 4 are, respectively, derived from groups of animals immunized i.d. with DNA for one, two, or three times in the presence of adjuvant. Lane 1 is a negative control of DNA from peritoneal lavage cells from animals injected with adjuvant only at both the i.d. and the i.p. sites. Quantities of total DNA were comparable for all samples, as determined by Southern blot analysis on PCR-amplified G3PDH gene (not shown).

group was sacrificed 3 days after 1 wk of immunization, another mouse 3 days after 2 wk of immunization, and the last mouse 3 days after 3 wk of immunization. Total DNA was extracted from peritoneal lavage cells (site of secondary inflammation), and Balf2-specific PCR on DNA from peritoneal cells was followed by Southern blot with an internal probe for Balf2. Southern analysis showed that repeated i.d. administration of DNA at the primary inflammatory site led to its accumulation at the peritoneal inflammatory site (Fig. 4), but not at an unrelated distant site such as the skin of the tail (not shown).

CD11b⁺ cells mediate the DNA transport between distant inflammatory sites

Because CD11b⁺ cells at the primary site of inflammation associated with uptake of DNA (not shown), we tested whether CD11b⁺ cells could transport DNA to distant sites. To this aim, we used a novel strategy that is modification of a technique described by Patterson et al. (26). Briefly, peritoneal lavage cells and PMBC were harvested from four individual DNA-immunized mice and labeled with anti-CD11b mAb. After cell sorting by flow cytometry, cells were fixed and permeabilized. Individual cells positive for the i.d. injected Ag were visualized by flow cytometry after Balf2-specific PCR-FISH (Fig. 6). This analysis, performed on

FIGURE 5. Balf2-PCR on total DNA of PEC of mice receiving or not the adjuvant at the peritoneal site concomitant with the i.d. DNA injection after three immunizations. The dashed arrow points at Balf2, the plain arrow at the housekeeping G3PDH. Lane 1, Molecular weight marker; lane 2, Balf-2 PCR on PEC from mice injected with adjuvant and not DNA; lane 3, Balf2-PCR on PEC from DNA-immunized mice not receiving adjuvant at the distant site; lane 4, G3PDH-PCR on PEC of lane 3; lane 5, Balf2-PCR on PEC from genetically immunized mice receiving adjuvant at the distant site; lane 6, G3PDH-PCR on PEC of lane 5.

both CD11b⁺ and CD11b⁻ cells, showed that the majority of peritoneal cells positive for the Ag were CD11b⁺ (Fig. 6). Thus, CD11b⁺ cells can mediate transport of ingested DNA between distant and unrelated inflammatory sites.

Discussion

We show herein that association of local inflammation with i.d. delivery of DNA at the same site can affect transport of DNA beyond local lymph nodes to distant inflammatory sites. Intradermal DNA immunization in the presence of adjuvant resulted in

FIGURE 6. Single-cell PCR/FISH/FACS. *a*, The sensitivity of the method is shown. The G3PDH gene is used as a positive control. The negative control is a nonrelated, digoxigenin-labeled probe. *b* and *c*, Comparison of the positivity for plasmid DNA in peritoneal lavage cells sorted for CD11b. More CD11b⁺ (*b*, 10.3% positive) than CD11b⁻ (*c*, 2.1% positive) peritoneal lavage cells contain Balf2. The M1 marker for *b* and *c* is set on the fluorescence of an irrelevant digoxigenin-labeled probe.

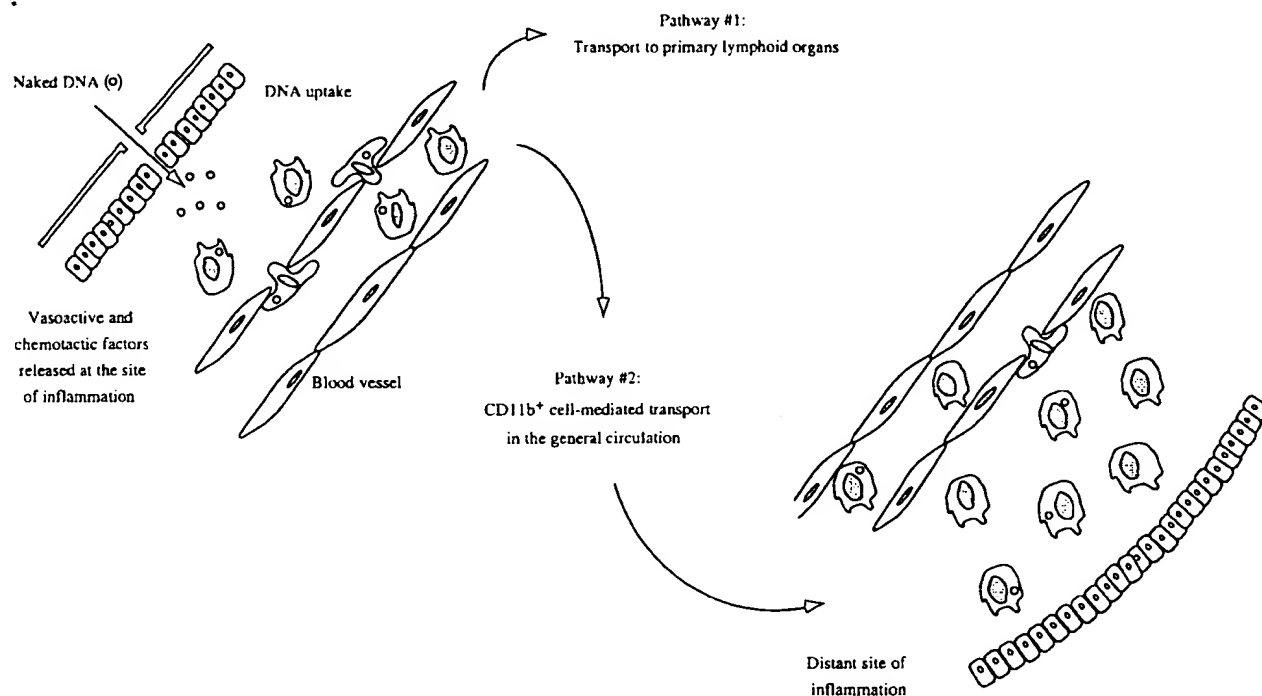


FIGURE 7. Schematic model suggesting the events occurring after naked DNA injection in the presence of inflammation.

local presence of plasmid DNA at the dermal site of inflammation and in situ expression of encoded proteic product, as similarly reported by others (12, 21). Immunofluorescence staining indicated intracellular Balf2 protein expression in cells of the dermis with the morphology of dendritic cells and in keratinocytes of the granular layer of epidermis. By coupling an inflammatory stimulus to i.d. injection of DNA, locally recruited phagocytic cells transported plasmid DNA beyond regional lymph nodes to distant sites such as the spleen. Furthermore, this cell-mediated transport of ingested DNA was also detected at a distant site of inflammation. Repeated administration of naked DNA at the primary inflammatory site led to its accumulation at the distant site of inflammation.

DNA vaccination induces broad-based, long-lasting, Ag-specific immune responses (2) and associates with in vivo transfection of phagocytic cells (10–15) that migrate to regional draining lymph nodes (21, 27, 28). Because of the capability of dendritic cells to uptake DNA and carry it to local lymph nodes, Condon et al. proposed to use these cells as vehicles for gene immunization (13). Also, Chattergoon et al. recently demonstrated the presence of transfected activated macrophages and dendritic cells in the blood and peripheral tissues of animals inoculated i.m. with DNA (22). Schubbert et al. found in mice that ingested foreign DNA passes the intestinal wall and can be found in distant organs such as the liver and the spleen (29). We extend the above information by showing that CD11b⁺ cells can mediate the transport of plasmid DNA between distant inflammatory sites after i.d. DNA immunization. The interpretation of our findings is schematically depicted in Fig. 7. DNA molecules are uptaken in the dermis by cells recruited at the inflammatory site. As indicated by confocal microscopy experiments, the injected DNA leads to relevant Ag expression in the host cells. CD11b⁺ cells that contain plasmid DNA migrate beyond lymph nodes into the general circulation and can reach distant organs such as the spleen or distant inflammatory sites, possibly attracted by a gradient of chemotactic stimuli (30–33).

Interesting correlations can be found between the DNA transport and the handling of proteic Ags by the immune system. In-

deed, in both cases 1) bone marrow-derived cells can actively uptake the molecule; 2) direct uptake of DNA by phagocytic cells may result in presentation of the encoded product to MHC-restricted T cells and generation of Ag-specific immune responses; 3) uptake of the molecule is increased by inflammation; and 4) transport is cell mediated and amplified by inflammation at the site of transport. These similarities may suggest the possibility that this pathway of transport can be relevant to both DNA and proteic Ags.

Our data also imply the possibility to manipulate abnormal immune responses at inflammatory sites. Different Ag-targeting strategies have been devised to specifically direct the Ag to APC to promote "site-directed immunogenesis" and obtain an effective immune response to DNA vaccines (34). Also in consideration of the findings of Song et al., who reported the suppression of ongoing inflammation in arthritis after i.m. injection of a plasmid-encoding TGF- β 1 (35), we can hypothesize that, by associating inflammatory stimuli with injection of naked DNA encoding for a given soluble mediator (or for an Ag such as for example a tolerogenic peptide), one might possibly obtain the transport of the relevant molecule to a distant inflammatory site and its in situ expression. Additional studies are required to address this possibility and the relevance of this pathway to chronic inflammation associated with infection.

Acknowledgments

We thank Drs. Dennis Carson, Francis Chisari, Carlos Carrera, and Jayne Leslie for critical reading of the manuscript, and Nancy Noon for secretarial assistance.

References

1. Pisetsky, D. S. 1996. The immunological properties of DNA. *J. Immunol.* 156: 421.
2. Ulmer, J. B., J. J. Donnelly, S. E. Parker, G. H. Rhodes, P. L. Felgner, V. J. Dwarki, S. W. Gromkowski, R. Randall Deck, C. M. DeWitt, A. Friedman, et al. 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science* 259:1745.
3. Michel, M. L., H. L. Davis, M. Schlee, M. Mancini, P. Tiollais, and R. G. Whalen. 1995. DNA-mediated immunization to the hepatitis B surface

- antigen in mice: aspects of the humoral response mimic hepatitis B viral infection in humans. *Proc. Natl. Acad. Sci. USA* 92:5307.
4. Donnelly, J. J., J. B. Ulmer, J. W. Shiver, and M. A. Liu. 1997. DNA vaccines. *Annu. Rev. Immunol.* 15:617.
 5. Feltquate, D. M., S. Heaney, R. G. Webster, and H. L. Robinson. 1997. Different T helper cell types and antibody isotypes generated by saline and gene gun DNA immunization. *J. Immunol.* 158:2278.
 6. Boyle, J. S., A. Silva, J. L. Brady, and A. M. Lew. 1997. DNA immunization: induction of higher avidity antibody and effect of route on T cell cytotoxicity. *Proc. Natl. Acad. Sci. USA* 94:14626.
 7. Robinson, H. L., and C. A. Torres. 1997. DNA vaccines. *Semin. Immunol.* 9:271.
 8. Yokoyama, M., D. E. Hassett, J. Zhang, and J. L. Whitton. 1997. DNA immunization can stimulate florid local inflammation, and the antiviral immunity induced varies depending on injection site. *Vaccine* 15:553.
 9. Danko, L., P. Williams, H. Herweijer, G. Zhang, J. S. Latendresse, I. Bock, and J. A. Wolff. 1997. High expression of naked plasmid DNA in muscles of young rodents. *Hum. Mol. Genet.* 6:1435.
 10. Corr, M., D. J. Lee, D. A. Carson, and H. Tighe. 1996. Gene vaccination with naked plasmid DNA: mechanism of CTL priming. *J. Exp. Med.* 184:1555.
 11. Robinson, H. L., S. Lu, D. M. Feltquate, C. A. T. Torres, J. Richmond, C. M. Boyle, M. J. Morin, J. C. Santoro, R. G. Webster, D. Montefiori, et al. 1996. DNA vaccines. *AIDS Res. Hum. Retroviruses* 12:455.
 12. Raz, E., D. A. Carson, S. E. Parker, T. B. Parr, A. M. Abai, G. Aichinger, S. H. Gromkowski, M. Singh, D. Lew, M. A. Yankauckas, et al. 1994. Intradermal gene immunization: the possible role of DNA uptake in the induction of cellular immunity to viruses. *Proc. Natl. Acad. Sci. USA* 91:9519.
 13. Condon, C., S. C. Watkins, C. M. Celluzzi, K. Thompson, and L. D. Falo. 1996. DNA-based immunization by in vivo transfection of dendritic cells. *Nat. Med.* 2:1122.
 14. Torres, C. A., A. Iwasaki, B. H. Barber, and H. L. Robinson. 1997. Differential dependence on target site tissue for gene gun and intramuscular DNA immunizations. *J. Immunol.* 158:4529.
 15. Iwasaki, A., C. A. Torres, P. S. Ohashi, H. L. Robinson, and B. H. Barber. 1997. The dominant role of bone marrow-derived cells in CTL induction following plasmid DNA immunization at different sites. *J. Immunol.* 159:11.
 16. Lu, B., G. Scott, and L. A. Goldsmith. 1996. A model for keratinocyte gene therapy: preclinical and therapeutic considerations. *Proc. Assoc. Am. Physicians* 108:165.
 17. Tighe, H., M. Corr, M. Roman, and E. Raz. 1998. Gene vaccination: plasmid DNA is more than just a blueprint. *Immunol. Today* 19:89.
 18. Steinman, R. M. 1991. The dendritic cell system and its role in immunogenicity. *Annu. Rev. Immunol.* 9:271.
 19. Larsen, C. P., R. M. Steinman, M. Witmer-Pack, D. F. Hankins, P. J. Morris, and J. M. Austyn. 1990. Migration and maturation of Langerhans cells in skin transplants and explants. *J. Exp. Med.* 172:1483.
 20. Doe, B., M. Selby, S. Barnett, J. Baenziger, and C. M. Walker. 1996. Induction of cytotoxic T lymphocytes by intramuscular immunization with plasmid DNA is facilitated by bone marrow-derived cells. *Proc. Natl. Acad. Sci. USA* 93:8578.
 21. Porgador, A., K. R. Irvine, A. Iwasaki, B. H. Barber, N. P. Restifo, and R. N. Germain. 1998. Predominant role for directly transfected dendritic cells in antigen presentation to CD8⁺ T cells after gene gun immunization. *J. Exp. Med.* 188:1075.
 22. Chattergoon, M. A., T. M. Robinson, J. D. Boyer, and D. B. Weiner. 1998. Specific immune induction following DNA-based immunization through in vivo transfection and activation of macrophages: antigen presenting cells. *J. Immunol.* 160:5707.
 23. Pollack, Y., R. Stein, A. Razin, and H. Cedar. 1980. Methylation of foreign DNA sequences in eukaryotic cells. *Proc. Natl. Acad. Sci. USA* 77:6463.
 24. Coligan, J. E., A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober. 1993. *Current Protocols in Immunology*. J. Wiley & Sons, New York.
 25. Hengge, U. R., P. S. Walker, and J. C. Vogel. 1996. Expression of naked DNA in human, pig, and mouse skin. *J. Clin. Invest.* 97:2911.
 26. Patterson, B. K., M. Till, P. Otto, C. Goolsby, M. R. Furtado, L. J. McBride, and S. M. Wolinsky. 1993. Detection of HIV-1 DNA and messenger RNA in individual cells by PCR-driven in situ hybridization and flow cytometry. *Science* 260:976.
 27. Kripke, M. L., C. G. Munn, A. Jeevan, J. M. Tang, and C. Bucana. 1990. Evidence that cutaneous antigen-presenting cells migrate to regional lymph nodes during contact sensitization. *J. Immunol.* 145:2833.
 28. Weinlich, G., M. Heine, H. Stossel, M. Zanella, P. Stoitzner, U. Ortner, J. Smolle, F. Koch, N. T. Sepp, G. Schuler, and N. Romani. 1998. Entry into afferent lymphatics and maturation in situ of migrating murine cutaneous dendritic cells. *J. Invest. Dermatol.* 110:441.
 29. Schubbert, R., D. Renz, B. Schmitz, B., and W. Doerfler. 1997. Foreign (M13) DNA ingested by mice reaches peripheral leukocytes, spleen, and liver via the intestinal wall mucosa and can be covalently linked to mouse DNA. *Proc. Natl. Acad. Sci. USA* 94:961.
 30. Issekutz, A. C., and T. B. Issekutz. 1992. The contribution of LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) to the in vivo migration of polymorphonuclear leukocytes to inflammatory reactions in the rat. *Immunology* 76:655.
 31. Issekutz, A. C., and T. B. Issekutz. 1995. Monocyte migration to arthritis in the rat utilizes both CD11/CD18 and very late activation antigen 4 integrin mechanisms. *J. Exp. Med.* 181:1197.
 32. Tang, T., A. Rosenkranz, K. J. M. Assmann, M. J. Goodman, J.-C. Gutierrez-Ramos, M. C. Carroll, R. S. Cotran, and T. N. Mayadas. 1997. A role for Mac-1 (CD11b/CD18) in immune complex-stimulated neutrophil function in vivo: Mac-1 deficiency abrogates sustained Fc receptor-dependent neutrophil adhesion and complement-dependent proteinuria in acute glomerulonephritis. *J. Exp. Med.* 186:1853.
 33. Johnston, B., T. B. Issekutz, and P. Kubes. 1996. The α_4 -integrin supports leukocyte rolling and adhesion in chronically inflamed postcapillary venules in vivo. *J. Exp. Med.* 183:1995.
 34. Langermann, S. 1998. Site-directed immunogenesis. *Nat. Med.* 4:547.
 35. Song, X., M. Gu, W. Jin, D. M. Klinman, and S. M. Wahl. 1998. Plasmid DNA encoding transforming growth factor- β 1 suppresses chronic disease in a streptococcal cell wall-induced arthritis model. *J. Clin. Invest.* 101:2615.